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Cell-cycle arrest and inhibition of Cdk4 activity by small peptides based on the carboxy-terminal domain of p21^{WAF1}

Kathryn L. Ball*, Sonia Lain†, Robin Fåhræus*, Carl Smythe‡ and David P. Lane*

Background: A common event in the development of human neoplasia is the inactivation of a damage-inducible cell-cycle checkpoint pathway regulated by p53. One approach to the restoration of this pathway is to mimic the activity of key downstream effectors. The cyclin-dependent kinase (Cdk) inhibitor p21^{WAF1} is one such molecule, as it is a major mediator of the p53-dependent growth-arrest pathway, and can, by itself, mediate growth suppression. The primary function of the p21^{WAF1} protein appears to be the inhibition of G1 cyclin–Cdk complexes. Thus, if we can identify the region(s) of p21^{WAF1} that contain its inhibitor activity they may provide a template from which to develop novel anti-proliferative drugs for use in tumours with a defective p53 pathway.

Results: We report on the discovery of small synthetic peptides based on the sequence of p21^{WAF1} that bind to and inhibit cyclin D1–Cdk4. The peptides and the full-length protein are inhibitory at similar concentrations. A 20 amino-acid peptide based on the carboxy-terminal domain of p21^{WAF1} inhibits Cdk4 activity with a concentration for half-maximal inhibition ($I_{0.5}$) of 46 nM, and it is only four-fold less active than the full-length protein. The length of the peptide has been minimized and key hydrophobic residues forming the inhibitory domain have been defined. When introduced into cells, both a 20 amino-acid and truncated eight amino-acid peptide blocked phosphorylation of the retinoblastoma protein (pRb) and induced a potent G1/S growth arrest. These data support a physiological role for the carboxyl terminus of p21^{WAF1} in the inhibition of Cdk4 activity.

Conclusions: We have discovered that a small peptide is sufficient to mimic p21^{WAF1} function and inhibit the activity of a critical G1 cyclin–Cdk complex, preventing pRb phosphorylation and producing a G1 cell-cycle arrest in tissue culture cell systems. This makes cyclin D1–Cdk4 a realistic and exciting target for the design of novel synthetic compounds that can act as anti-proliferative agents in human cells.

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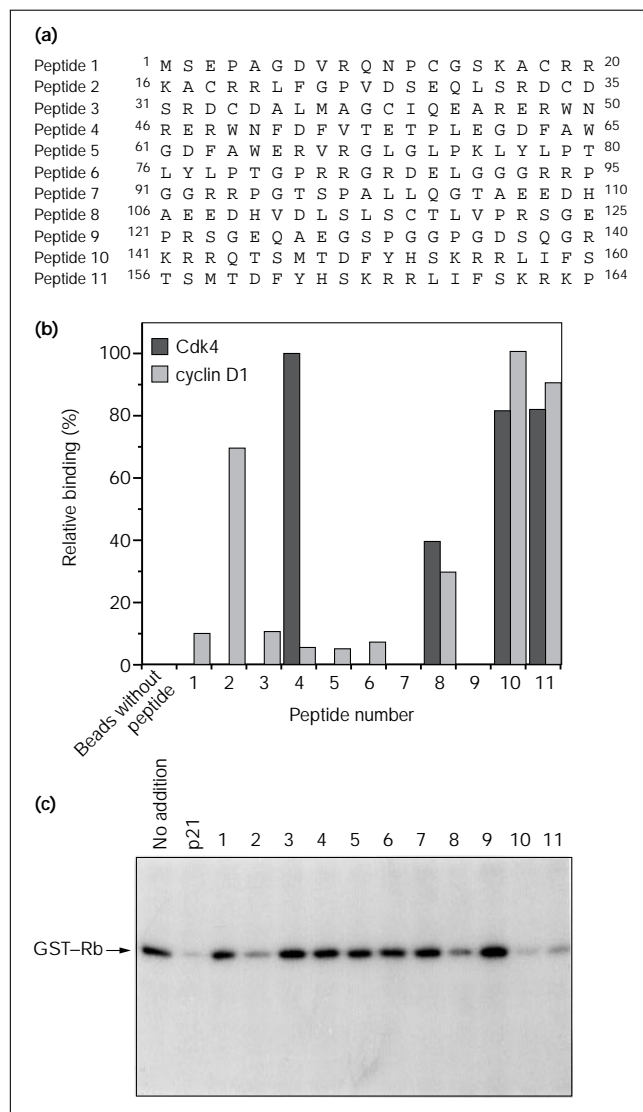
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Background

Tumour suppression by p53 is linked to a cell-cycle checkpoint induced by DNA damage [1], in which p53 can induce either growth arrest [2] or apoptosis [3–5] in the damaged cells. The biochemical activity of p53 most tightly associated with tumour suppression and growth arrest involves a damage-dependent activation of sequence-specific transcriptional activity [1,6,7]. p53 induces the transcription of a number of genes, the products of which directly mediate growth arrest. These p53-inducible negative regulators of cell proliferation include: the cyclin dependent kinase (Cdk) inhibitor (CKI), p21^{WAF1} [8–11]; an apoptosis-promoting protein, Bax [12]; the insulin growth factor binding protein, IGF-BP3 [13]; and Gadd45 [14], a potent inhibitor of cell proliferation with an as yet unclear biochemical function [15].

Inactivation of the damage-inducible cell-cycle checkpoint pathway regulated by p53 is a common event in the development of human neoplasia [16,17]. A variety of mechanisms can lead to the functional inactivation of the p53 pathway, including missense mutations or deletions of the p53 gene, inactivation of wild-type p53 protein function by interaction with the oncogenic cellular protein mdm-2 [18], or the inability to induce downstream effector molecules, such as p21^{WAF1} [19,20]. Our growing knowledge of the molecular mechanisms underlying the transformation of mammalian cells offers the opportunity to create rationally designed inhibitors of specific biochemical processes essential to cell proliferation or cancer. Recent developments have shown that the reactivation of the p53 pathway in some human tumours could in theory be achieved by several means. The biochemical function

Figure 1



The ability of peptides from p21^{WAF1} to interact with and inhibit cyclin D1–Cdk4. **(a)** A list of the peptides (1–11) based on the sequence of p21^{WAF1}. **(b)** The p21^{WAF1} peptides were bound to streptavidin-agarose beads and added to reticulocyte lysates containing either Cdk4 or cyclin D1 labelled with [³⁵S]methionine. After extensive washing, bound proteins were analysed using SDS-PAGE followed by autoradiography. The bands were quantified using a Bio-Imager and Whole Band Analysis Software (Millipore). The results are representative of three such experiments. **(c)** Cyclin D1–Cdk4 kinase assays were carried out *in vitro* using lysates from Sf9 insect cells following co-infection with Cdk4 and cyclin D1 baculovirus constructs and using GST–Rb as the substrate. p21^{WAF1} peptides were added to the assays at a concentration of 17 μ M, and the effect on Cdk4 activity was assessed by SDS-PAGE and autoradiography. The data are representative of four experiments.

of mutant p53 protein could be activated [21,22], possibly using small peptides as leads for drug design [23]. The interaction of the oncogene mdm-2 and wild-type p53 could be disrupted through the use of peptidomimetic inhibitors of complex formation [24]. Finally, the function

of the downstream effector molecule p21^{WAF1}, which on its own is capable of mediating growth suppression [8,25], could be restored or mimicked.

The p21^{WAF1} protein appears to act during growth suppression primarily as an inhibitor of the G1 cyclin–Cdk complexes [26–29]. Here, we describe functional studies of the interaction of small peptides derived from p21^{WAF1} with cyclin D1–Cdk4 complexes. This cyclin–Cdk holoenzyme complex was chosen because the D-type cyclins are required for cell-cycle progression in G1 phase [30,31], and inhibition of the cyclin D-associated kinase Cdk4 (along with its isoform Cdk6) leads to an arrest of the cell cycle in G1 phase [32]. Thus, the cyclin D1–Cdk4 complex is a good target for the development of compounds aimed at preventing cell proliferation.

With the aim of identifying low molecular weight peptide mimetics of full-length p21^{WAF1} protein that could function in cells, we carried out a systematic study to examine the binding and inhibitory properties of a library of synthetic peptides based on the amino-acid sequence of p21^{WAF1}. These studies have led to the identification of low molecular weight mimetics of the p21^{WAF1} protein that act as growth inhibitors *in vivo*. These data have exciting implications for the mechanism of action of p21^{WAF1} protein and represent a starting point for a drug design programme aimed at producing small synthetic molecules that function as tumour suppressors downstream of p53.

Results

Peptides from the carboxyl terminus of the p21^{WAF1} protein are potent inhibitors of Cdk4 activity

A major advance in research aimed at replacing downstream effectors of p53 activity in human tumour cells would be to produce low molecular weight mimetics of p21^{WAF1}. Using a series of synthetic peptides that span the entire sequence of the p21^{WAF1} protein (Fig. 1a), we determined whether any of these peptides could mimic full-length p21^{WAF1} protein *in vitro* by assaying for binding to cyclin D1 and Cdk4, and for inhibition of Cdk4 catalytic activity.

Using the previously described peptide precipitation assay [32], we identified three domains of p21^{WAF1} that form stable complexes with cyclin D1 and/or Cdk4 (Fig. 1b). Domain 1 consists of peptide 2, which binds stably only to cyclin D1 and contains the LeuPheGly motif essential for the interaction of the p21^{WAF1} family with cyclins [33–35]. Domain 2 consists of peptide 4, which only binds stably to Cdk4 and spans a region of p21^{WAF1} implicated in Cdk2 interactions [35–38]. Domain 3 comprises peptides 10 and 11, which have not been shown previously to be involved in direct binding to cyclin D1 or Cdk4.

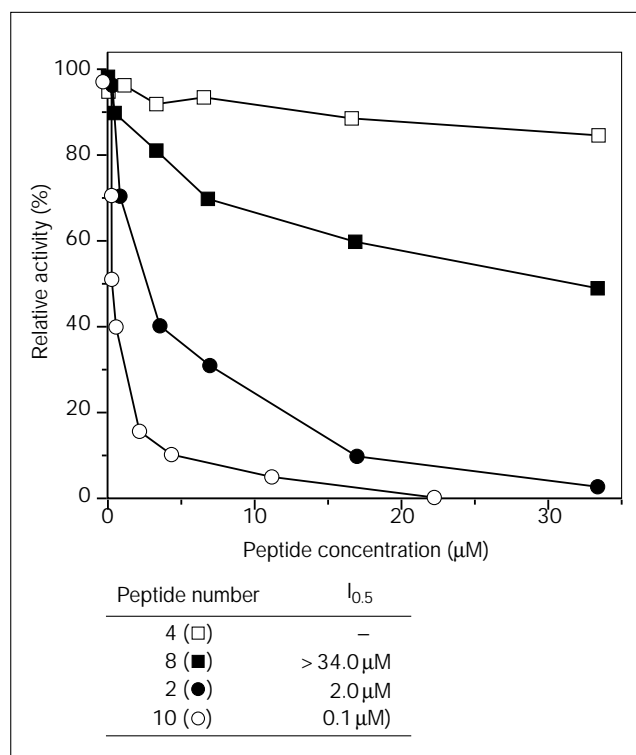
The binding of domain 1 and domain 2 peptides (Fig. 1b) are consistent with previously published physiological

data showing that the amino-terminal half of the p21^{WAF1} protein is required for binding to the cyclin–kinase complex [26,28,34]. This is, however, the first time that small peptides from the amino terminus have been shown to interact directly with either cyclin or Cdk, thus establishing that these regions of the p21^{WAF1} protein are not only necessary but sufficient for stable binding to either cyclin D1 (domain 1) or Cdk4 (domain 2). Strikingly, domain 3 peptides define a region from the carboxyl terminus of p21^{WAF1} that has not previously been shown to play a physiological role in the regulation of cyclin D1–Cdk4. This peptide domain differs from the other two in that it appears to interact with both cyclin D1 and Cdk4 (Fig. 1b).

The p21^{WAF1} peptides that bind to the cyclin or Cdk subunits may mimic the inhibitory activity of full-length p21^{WAF1}, providing a model for the design of p21^{WAF1} mimetics. The p21^{WAF1} peptides were therefore also tested independently for inhibition of Cdk4 kinase to determine if binding of peptides correlated with inhibitory function. Using the retinoblastoma protein (pRb)-phosphorylation assay, we identified two domains of p21^{WAF1} with inhibitory activity (Fig. 1c). The inhibition of Cdk4 activity by peptide 2 (domain 1) correlates with its ability to interact stably with cyclin D1 (Fig. 1b), and suggests there may be an allosteric component to Cdk4 regulation (our unpublished observations). These data are also consistent with previous reports showing that the amino-terminal half of p21^{WAF1} is required for kinase inhibition [26,28,34,37]. In addition, the pronounced inhibition by peptides 10 and 11 (domain 3) defines a potent Cdk4 inhibitory region. This inhibition also correlates with the ability of these peptides to bind to cyclin and kinase subunits (Fig. 1b). Interestingly, although we have identified a small peptide with a high binding affinity for Cdk4 (domain 2), it has no inhibitory activity when added to kinase assays at concentrations of up to 35 μ M. This suggests that compounds that mimic p21^{WAF1} by binding to the Cdk4 subunit may not prove to be good templates for the design of anti-proliferative drugs.

A titration of peptides from domain 1 (peptide 2), domain 2 (peptide 4), and domain 3 (peptide 10) was performed to determine their relative potency as kinase inhibitors (Fig. 2). The carboxy-terminal domain (peptide 10) had the highest activity, with a concentration for half-maximal cyclin D1–Cdk4 inhibition ($I_{0.5}$) of 0.1 μ M. Based on these biochemical studies, peptide 10 defines a potent lead for the design of small synthetic peptides that bind to and inhibit cyclin D1–Cdk4 kinase. The remainder of this report describes the characterization of this peptide *in vitro* and studies to determine whether the peptide has biological activity as an inhibitor of cellular proliferation.

Figure 2



Quantification of inhibition by peptides. Peptides 2 (●), 4 (□), 8 (■) and 10 (○) were added to cyclin D1–Cdk4 assays at various concentrations between 0.01 and 34 μ M, and GST–Rb phosphorylation was measured. At the top is a plot of the percentage activity (relative to Cdk4 activity measured in the absence of peptide) against peptide concentration. At the bottom, the $I_{0.5}$ for each peptide is tabulated. The data represent the mean of three experiments.

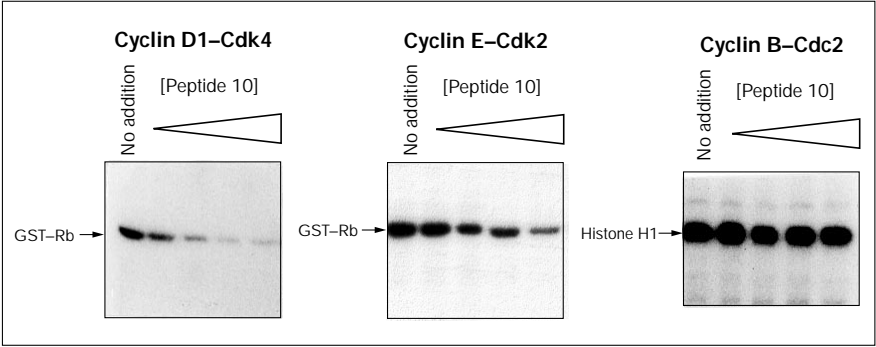
Carboxy-terminal peptide 10 inhibits cyclin D1–Cdk4 specifically

p21^{WAF1} displays selectivity for the G1- and S-phase cyclin–Cdk complexes [27]. We examined the specificity of peptide 10 by determining its $I_{0.5}$ for the inhibition of cyclin E–Cdk2 and cyclin B–Cdc2. When added to kinase assays at concentrations up to 20 μ M, peptide 10 had no significant effect on cyclin B–Cdc2 histone H1 kinase activity (Fig. 3). It did, however, inhibit the activity of cyclin E–Cdk2. The $I_{0.5}$ for the inhibition was 4 μ M, which is ~40-fold higher than the $I_{0.5}$ for cyclin D1–Cdk4. Thus, the p21^{WAF1}-based inhibitor, peptide 10, has a much higher specific activity against cyclin D1–Cdk4 than cyclin E–Cdk2 and, in common with full-length p21^{WAF1} protein, is a very poor inhibitor of cyclin B–Cdc2.

Identification of the inhibitory motif and minimization of the lead peptide

To produce a realistic template for the design of novel anti-proliferative drugs, we wished to determine if the length of our inhibitory peptide could be significantly reduced whilst retaining some inhibitory activity. Residues

Figure 3

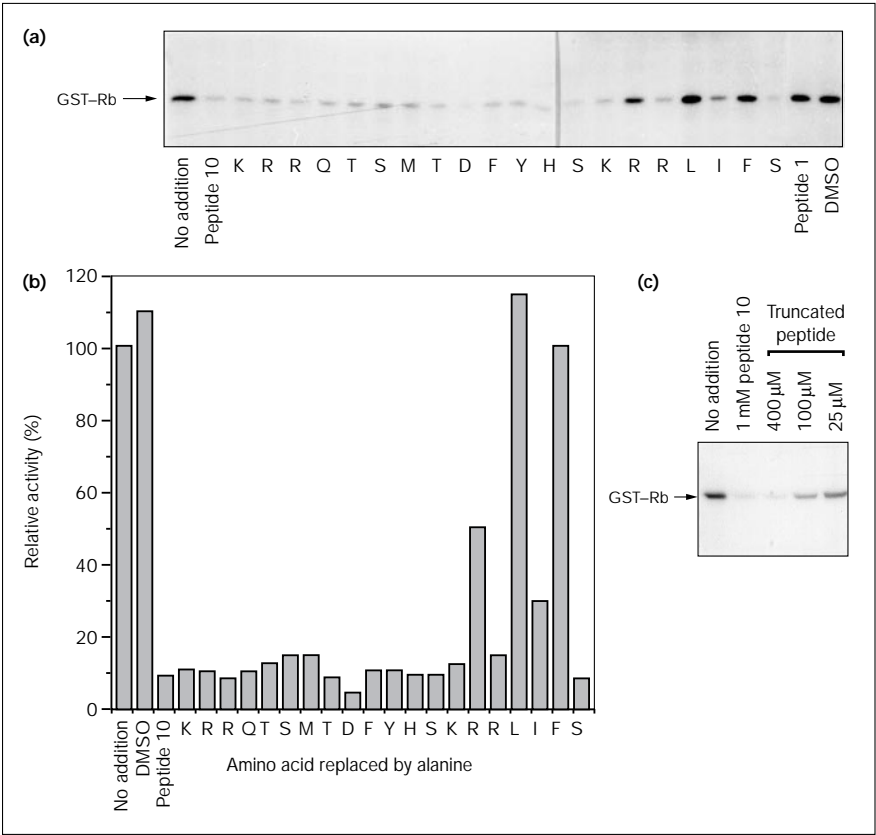


The inhibition by peptide 10 is specific to certain cyclin-Cdk complexes. The ability of peptide 10 to inhibit the activity of other cyclin-Cdk enzymes was assessed using cyclin E-Cdk2 and cyclin B-Cdc2. The kinase activity assays were performed using Sf9 cell lysates, which were co-expressing either human cyclin E and Cdk2, or human cyclin B and Cdc2. The conditions were identical to those described for cyclin D1-Cdk4 assays, except that histone H1 (0.5 mg per assay) was used as a substrate where indicated. The assays were carried out in the presence of increasing concentrations of peptide 10 (0.1, 0.5, 5, 20 μ M).

that were required for peptide function were first identified using a series of peptides, based on peptide 10, in which each residue was sequentially mutated to alanine (alanine scan; Fig. 4). We identified three residues, within a stretch of just five amino acids, as being extremely important for activity. The five amino acids formed an inhibitory motif, **RRLIF** (amino acids 155–160, in the single-letter amino-acid code), where the residues

in bold are essential for activity and the underlined residue contributes towards inhibitory activity. The fact that a single point mutation in either of two hydrophobic residues (the leucine or phenylalanine residues) completely abolishes inhibitory activity suggested that inhibition was the result of a specific interaction at key hydrophobic residues. In addition, these data establish that the carboxy-terminal Cdk4-inhibitory motif and the

Figure 4



Alanine-scan mutations and minimization of the length of peptide 10. (a) To pinpoint residues that are critical for the inhibition of Cdk4 by peptide 10, a series of point mutations were constructed in which each residue was sequentially changed to alanine. The peptides were added to cyclin D1-Cdk4 assays and the results were analysed by SDS-PAGE and autoradiography before (b) quantification using a Bio-Imager. The results are expressed relative to Cdk4 activity in the absence of peptide and are representative of three experiments. (c) Having identified the critical residues we then synthesized an untagged eight amino-acid peptide (KRRLIFSK) that contains the critical arginine, leucine and phenylalanine residues. The phosphorylation of GST-Rb by cyclin D1-Cdk4 in the presence of various concentrations of this truncated peptide are shown.

PCNA-binding motif of p21^{WAF1} [39,40] are distinct from one another.

Having identified residues that appeared to be critical for the inhibition of cyclin D1–Cdk4 by peptide 10, we determined if these residues were in fact sufficient for inhibition, or if they had to be presented within the context of a larger peptide. Strikingly, the eight amino-acid peptide, KRRLIFSK, retained the ability completely to inhibit cyclin D1–Cdk4 activity and prevent phosphorylation of pRb (Fig. 4). The $I_{0.5}$ for the truncated peptide was, however, ~1000-fold higher than that of the full-length peptide ($I_{0.5}$ for the truncated peptide was ~100 μ M). This was not an unexpected result, as other studies have shown loss of potency upon reducing the length of bioactive peptides. It may, however, be possible to improve the peptide inhibitory activity by manipulating the non-essential residues in a manner defined by Li *et al.* [41] in an elegant series of experiments aimed at minimizing the length of the atrial natriuretic peptide.

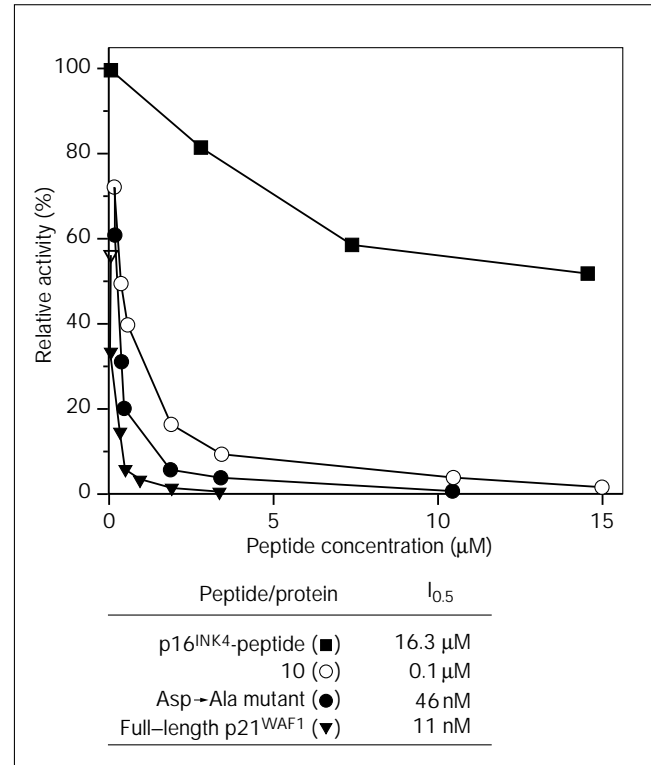
A substituted peptide 10 approaches the specific activity of full-length p21^{WAF1}

Whilst carrying out the alanine scan experiments, we noticed that one of the mutant peptides (Asp→Ala at position 9 of peptide 10; Fig. 4) was a better inhibitor of Cdk4 activity than the wild-type peptide. We determined the $I_{0.5}$ for this peptide and compared it with that of peptide 10, full-length purified p21^{WAF1}, and a peptide derived from the tumour suppressor protein p16^{INK4} that has been reported to inhibit Cdk4 activity *in vitro* and to prevent cell-cycle progression [32]. The Asp→Ala mutation decreases the $I_{0.5}$ of the full-length peptide 10 from 100 nM to 46 nM (Fig. 5). Comparing this with the p16^{INK4}-based peptide, which has an $I_{0.5}$ of 16.3 μ M (Fig. 5), reveals that we have produced a peptide that is ~350-fold more active than the p16^{INK4}-based peptide as a Cdk4 inhibitor. In fact, this peptide functions within the physiological range of p21^{WAF1} itself, which has an $I_{0.5}$ of 11 nM (Fig. 5). We do not know why the substitution of alanine for this aspartic acid, which is well outside the domain shown to be essential for activity, reduces the $I_{0.5}$. It seems likely that the altered residue changes the presentation of the inhibitory motif rather than functioning directly in inhibition, as this mutation does not appear to increase the affinity of the peptide for either Cdk4 or cyclin D1 (data not shown). Thus, we have now designed a peptide that has only a four-fold lower specific activity than full-length p21^{WAF1}.

Inhibition of proliferation and pRb phosphorylation in human cells

We and others have recently shown that a 16 amino-acid sequence from the homeodomain of the Antennapedia protein can act as a carrier for peptides with biological activity, translocating them across the plasma membrane and allowing them to interact with their target molecules

Figure 5



Comparison of inhibitory peptides with full-length p21^{WAF1} protein. Top: concentration curves for peptide 10 (○), Asp→Ala mutant peptide 10 (●), a p16^{INK4}-derived peptide (■) [32] and full-length p21^{WAF1} (▼) were determined using the cyclin D1–Cdk4 assay analysed by SDS-PAGE, autoradiography and bio-imaging. Bottom: the $I_{0.5}$ of each inhibitor. The results are the mean of three such experiments.

[32,42]. To determine if peptide 10 retains its biological activity when introduced into tissue culture cells, a chimaeric peptide, containing both carrier and peptide 10 sequences, was synthesized and added to a culture of proliferating asynchronous human-keratinocyte-derived HaCaT cells, which are mutant for p53 [43]. The linked peptide (peptide I; Fig. 6) contained a Met→Ala mutation at position 7; this abolished any PCNA binding function [39,40], and allowed us to study the PCNA-independent effects of the peptide in cells.

Peptide I was added to the culture media at a concentration of 25 μ M. The cells were fixed 24 hours later and analysed by fluorescence-activated cell sorting. Figure 6b,c shows the cell-cycle distribution of untreated and peptide I-treated cells assayed using bromodeoxyuracil (BrdU). The number of cells entering S phase in the presence of peptide I was dramatically reduced as compared with the control, and the number of cells in G1 showed a concomitant increase. This suggests that peptide I mimics the ability of full-length p21^{WAF1} to act as a growth suppressor by inducing a G1 cell-cycle arrest. Similar studies with human

in the presence of peptide I pRb remained hypophosphorylated. In addition, when peptide 10 was added to synchronous HaCaT cells 14 hours after serum stimulation (as the cells were passing through the restriction point at which pRb becomes hyperphosphorylated) its effect on cell-cycle distribution was greatly diminished (data not shown). Together, these data indicate that peptide I causes a G1 arrest in human HaCaT cells by preventing the phosphorylation of pRb.

In similar experiments with proliferating HaCaT cells, we introduced the eight-amino-acid bioactive derivative of peptide 10 (Fig. 6d; peptide II), and a control peptide 10 that lacked the hydrophobic residues which are essential for Cdk4 inhibition (Fig. 6e; peptide III). We found that peptide II effectively promoted a G1-phase arrest and prevented the phosphorylation of pRb when added at 50 μ M (Fig. 10h). Peptide III, however, had no significant effect on the ability of HaCaT cells to enter S phase. It is interesting that the truncated peptide 10, when coupled to carrier peptide (peptide II) and introduced into cells, is only 2–4-fold less active as a growth suppressor than peptide I. Linking the truncated peptide 10 to the carrier peptide may promote a more favourable inhibitory conformation, as the $I_{0.5}$ for the carrier-linked truncated peptide (peptide II) *in vitro* is ~50-fold less than that of the free truncated peptide (data not shown).

Discussion

Identification of a physiologically relevant Cdk4 regulatory site from the carboxyl terminus of p21^{WAF1}

Synthetic peptides or peptidomimetics are proving to be useful in studying the biochemical regulation of enzymes and proteins, and also in providing models for the design of novel anti-proliferative agents targeted to the proteins or enzymatic pathways that are altered or activated in human tumours [44,45]. Several peptides have been shown effectively to target components of the cell-cycle machinery. These include farnesyl-transferase inhibitor (FTI), which inhibits farnesyl protein transferase preventing the activation of Ras [46], Ras effector domain peptides, which can inhibit the biological function of Ras [47,48], polypeptides containing SH2/SH3 domains, which in theory should inhibit the growth of tumours with activated tyrosine kinases [49,50], and p16^{INK4}-derived peptides, which inhibit cyclin D–Cdk complex activity and thereby activate pRb-dependent cell-cycle arrest [32].

Inactivation of the tumour suppressor protein p53 is a common event in the development of human neoplasia [16]. The p53 protein is a key player in an inducible cell-cycle checkpoint pathway activated in response to DNA damage and perturbation of nucleotide pools [2,17]. Reactivation of this pathway by the restoration of p53 function could therefore provide a route to a novel anti-proliferative therapy. In general, however, the pharmacological

restoration of biochemical function to a protein that has lost its normal activity through mutation of its amino-acid sequence is more difficult than the inhibition of a biochemical function [45]. Thus, it may prove more productive to take alternative approaches to restore activity to the p53 pathway, such as mimicking the inhibitory activity of the downstream effector molecule p21^{WAF1}, which can by itself mediate growth arrest primarily through its interaction with the G1 cyclin–Cdk complexes [8,25,27].

We have identified a region from the carboxyl terminus of p21^{WAF1} that is important for interactions with G1 cyclin–Cdk complexes, and we are using this region as a template to design novel anti-proliferative agents. A peptide based on this region bound to and potentially inhibited cyclin D1–Cdk4 *in vitro*, and retained biological activity when introduced into proliferating cells. A peptide composed of amino acids 141–160 of p21^{WAF1} inhibited Cdk4 activity with an $I_{0.5}$ of 0.1 μ M, and caused cycling cells to arrest at the G1/S-phase boundary. In addition, we have shown that there is scope for producing peptides with improved activity, as mutations introduced into peptide 10 have reduced its $I_{0.5}$ to 46 nM. This compares favourably with the $I_{0.5}$ of full-length p21^{WAF1} protein (11 nM).

Peptide 10 represents a potentially exciting lead for rational drug design, as it is by far the most potent peptide inhibitor of Cdk activity discovered to date, with the Asp→Ala mutant peptide being ~350-fold more potent than the recently identified p16^{INK4} peptide [32]. The fact that the peptide retains biological activity when reduced to just eight amino acids improves its appeal as a template for drug design. In general, protein–protein interfaces are relatively large, relying on the participation of 10–30 contact sidechains on each interface, with each region of contact often being composed of residues that are dispersed throughout the primary amino-acid sequence [51,52]. There is evidence, however, that in some cases only a small subset of these sidechains need to be contacted for efficient binding to occur [53,54]. Our discovery that a single eight amino-acid peptide is sufficient to inhibit the activity of a critical G1 cyclin–Cdk complex, preventing pRb phosphorylation and producing a G1 cell-cycle arrest in tissue-culture cells, suggests that interaction at only a small subset of contact sidechains is necessary for potent inhibition of cyclin D1–Cdk4 activity at the G1–S phase boundary. This makes cyclin D1–Cdk4 a realistic and exciting target for the design of small synthetic compounds that can act as anti-proliferative agents.

Specificity of p21^{WAF1} peptide leads for cyclin D1–Cdk4

Prior to our studies, no data on the direct interaction of small peptides with either cyclins or Cdks had been reported. In addition, no evidence existed to suggest that a small peptide derived from the p21^{WAF1} protein would in fact be biologically active as a Cdk inhibitor in cells. While

this manuscript was under review, Chen *et al.* [33] reported that a peptide based on the carboxyl terminus of the p21^{WAF1} protein could prevent the formation of a stable complex between p21^{WAF1} and cyclin E or A, and that this peptide was a weak inhibitor of Cdk2 activity *in vitro*. Although data were not presented showing that peptides could bind directly to cyclins or Cdks, it was proposed that the RRLI motif contained within peptide 10 represented a cyclin-interacting site comparable to that contained within peptide 2, RRLF. However, in our direct binding measurements we found that the mechanism of action of peptide 10 was different to that of peptide 2, as peptide 10 could bind to both cyclin D1 and Cdk4, whereas peptide 2 could only bind to cyclin D1 (similar results have also been obtained with cyclin D1 and Cdk4 expressed in *Escherichia coli*; data not shown). Although we agree that peptides from the carboxy-terminal region of p21^{WAF1} do not have a high specific activity for Cdk2 inhibition, they are potent inhibitors of Cdk4 activity.

The fact that Chen *et al.* [33] did not find peptides that competed with p21^{WAF1} for binding to cyclin D1 may be explained by their use of 12 amino-acid peptides. In the present study, we found that the length of peptide 10 was critical for stable binding. When non-critical residues were removed from the amino terminus of the peptide, its affinity for cyclin D1 and Cdk4 was greatly reduced. In fact, the increase in $I_{0.5}$ for the eight amino-acid truncated version of peptide 10 reflects its inability to form a stable complex with cyclin D1–Cdk4 (data not shown), suggesting that the inhibitory motif may be given a favourable inhibitory conformation by residues outside of the RRLIF motif.

We have defined the residues that are important for biochemical activity of the carboxy-terminal peptide from p21^{WAF1} and find that the leucine and phenylalanine, but not the isoleucine, are essential for both binding (data not shown) and inhibition (Fig. 4), indicating that the inhibitory binding motif is RRLIF. Our study points to the importance of carrying out detailed mechanistic studies on each cyclin–Cdk–CKI combination in order to define accurately the specificity of the CKI for each cyclin–Cdk complex. For example, removal of the amino-terminal 34 amino acids (containing the cyclin-binding motif found in peptide 2) from the *Xenopus* p21^{WAF1} homologue Xic1 results in a protein that is compromised in its ability to inhibit cyclin A–Cdk2, but is still an efficient inhibitor of cyclin E–Cdk2 activity [55]. In addition, the crystal structure of the amino-terminal half of p27^{KIP1} suggests that the association of one molecule of p27^{KIP1} with cyclin A–Cdk2 is inhibitory [35]. This is in contrast to the clear biochemical evidence that two molecules of the p21^{WAF1} protein are required for Cdk inhibition, with cyclin–Cdk complexes containing a single molecule of p21^{WAF1} being catalytically active [27,56].

Conclusion

These studies have led to the identification of small molecular weight mimetics of the p21^{WAF1} protein that act *in vivo* as growth inhibitors by preventing the phosphorylation of pRb. We have identified novel binding peptides that potently inhibit Cdk4 activity within the same physiological concentration range as full-length p21^{WAF1}. These data pinpoint a cyclin D1–Cdk4 inhibitory motif and represent a starting point for a drug-design programme aimed at producing small synthetic molecules functioning as tumour suppressors downstream of p53.

Materials and methods

Peptides

All biotinylated peptides were synthesized by Chiron Mimotopes, Peptide Systems (Clayton, Australia). Each peptide had a biotin-SGSG spacer at the carboxyl terminus and a free amino terminus. The peptides were dissolved in DMSO at ~5 mg ml⁻¹. We then determined their concentration precisely by amino-acid analysis. The purity of the peptides was estimated using mass spectrometry. The truncated peptide 10 and peptides for application to cells were kindly provided by Weng Chan; they were synthesized using standard Fmoc/tBu solid-phase chemistry and derivatized NovaSyn TG resin (Calbiochem-Novabiochem UK) on an automated peptide synthesizer (PepSynthesizer 9050; MilliGen/Bioscience UK). The peptides were purified by reverse-phase HPLC on a semi-preparative Hypersil Pep C18 column (Shandon HPLC, UK) using 0.06 % aqueous TFA/acetonitrile gradient elution. The purity (> 95 %) was established by HPLC and mass spectrometry (MS; either laser desorption-MS on a Kompact Maldi III; Kratos Analytical, or electrospray-MS on a VG Platform; Fison).

Proteins

Cyclins and Cdks for kinase assays were co-expressed in Sf9 insect cells infected with the appropriate baculovirus constructs. The cells were harvested two days after infection by low-speed centrifugation and the pellet was lysed in an equal volume of 10 mM Hepes, pH 7.4 containing 10 mM NaCl, 1 mM EDTA, 2 mM DTT and 0.1 mM phenylmethane sulphonyl fluoride, before centrifugation at 14 000 $\times g$ for 15 min. Labelled Cdk4 and cyclin D1 were produced by translation in the presence of [³⁵S]methionine using a rabbit reticulocyte lysate *in vitro* translation kit (Promega).

Human His-tagged p21^{WAF1} was expressed in *E. coli* using a PET expression vector. The soluble p21^{WAF1} protein fraction was purified using a nickel chelating column and gel filtration chromatography on a Superose 12 column, following the manufacturers instructions (Pharmacia).

GST–Rb was produced using an *E. coli* expression construct containing the hyperphosphorylation domain of pRb (amino acids 773–924). The protein was purified on a glutathione-Sepharose column according to the manufacturer's instructions (Pharmacia).

Peptide precipitation of Cdk4 and cyclin D1

Peptide precipitation assays were performed as described [32]. The bound protein was analysed by SDS-PAGE followed by auto-radiography and quantification of the ³⁵S-labelled protein using a Bio-Imager and Whole Band Analysis Software (Millipore).

Enzyme assays

Cdk phosphorylation of GST–Rb was measured using the insect-cell lysates described above. Extract (1 μ l) was added to a final reaction volume of 10 μ l, containing 50 mM Hepes, pH 7.4, 10 mM MgCl₂, 2.5 mM EGTA, 1 mM DTT, 10 mM β -glycerophosphate, 1 mM NaF, 10 mM PKI (protein kinase inhibitor) 50 μ M ATP containing [³²P]ATP (1000 cpm pmol⁻¹). The assays were started by the addition of 0.5 μ g of the substrate (GST–Rb or histone H1), incubated at 30 °C for 10 min,

and terminated by adding SDS-PAGE sample buffer and heating at 95 °C for 4 min. The samples were analysed on 12 % SDS-PAGE gels followed by autoradiography and quantification using a Bio-Imager.

Cell-cycle measurements

Carrier-linked peptides were designed (Fig. 6a) for delivery into proliferating HaCaT cells. Cells were seeded on 30-mm-diameter culture plates and grown to 50 % confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) foetal calf serum (FCS). Peptides were added to the medium and the cells were incubated for 24 h. During the last 30 min of the incubation, the cells were pulse-labelled in the presence of 15 mM BrdU. The cells were trypsinized, fixed in absolute alcohol and prepared for analysis by FACS using a single-laser flow cytometer (Becton-Dickinson) [57].

pRb phosphorylation in HaCaT cells

HaCaT cells were seeded on 30-mm-diameter culture plates at 25 % confluency in DMEM with 10 % FCS. The FCS was withdrawn after 24 h and the cells were starved for 72 h. At the end of this period the medium was supplemented with 10 % FCS and carrier-linked peptides. Samples were taken over a 24 h time course by lysing the cells in RIPA buffer. The phosphorylation status of pRb was determined by western-blot analysis [32]; the blot was probed with the anti-pRb polyclonal antibody C-15 (Santa Cruz).

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References

- Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW: Participation of p53 protein in the cellular response to DNA damage. *Cancer Res* 1991, 51:6304–6311.
- Agarwal M, Agarwal A, Taylor W, Stark G: p53 controls both the G₂/M and the G₁ cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc Natl Acad Sci USA* 1995, 92:8493–8497.
- Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML, Wyllie AH: Thymocyte apoptosis induced by p53 dependent and independent pathways. *Nature* 1993, 362:849–852.
- Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T: p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* 1993, 362:847–849.
- Merritt AJ, Potten CS, Kemp CJ, Hickman J, Balmain A, Lane DP, Hall PA: The role of p53 in spontaneous and radiation-induced apoptosis in the gastrointestinal tract of normal and p53-deficient mice. *Cancer Res* 1994, 54:614–617.
- Lu X, Lane DP: Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes. *Cell* 1993, 75:765–778.
- Pietenpol JA, Tokino T, Thiagalingam S, El-Deiry W, Kinzler K, Vogelstein B: Sequence-specific transcriptional activation is essential for growth suppression by p53. *Proc Natl Acad Sci USA* 1994, 91:1998–2002.
- El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, *et al.*: WAF-1, a potential mediator of p53 tumor suppression. *Cell* 1993, 75:817–825.
- Harper JW, Adami GR, Wei N, Keyomasi K, Elledge SJ: The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 1993, 75:805–816.
- Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D: p21 is a universal inhibitor of cyclin kinases. *Nature* 1993, 366:701–704.
- Gu Y, Turck CT, Morgan DO: Inhibition of Cdk2 activity *in vivo* by an associated 20K regulatory subunit. *Nature* 1993, 371:257–261.
- Miyashita T, Reed JC: Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* 1995, 80:293–299.
- Buckbinder L, Talbot R, Velasco-Miguel S, Takenaka I, Faha B, Siezinger BR, Kley N: Induction of the growth inhibitor IGF-binding protein 3 by p53. *Nature* 1995, 377:646–649.
- Kastan MB, Zhan Q, El-Deiry WK, Carrier F, Jacks T, Walsh WV, *et al.*: A mammalian cell cycle checkpoint pathway utilising p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* 1992, 71:587–597.
- Kearsey JM, Shivji MKK, Hall PA, Wood RD: Does the p53 up-regulated Gadd45 protein have a role in excision repair? *Science* 1995, 270:1004–1005.
- Hollstein M, Sidransky D, Vogelstein B, Harris C: p53 mutations in human cancer. *Science* 1991, 253:49–53.
- Lane DP: p53, guardian of the genome. *Nature* 1992, 358:15–16.
- Momand J, Zambetti GP, Olson DC, Levine AJ: The mdm-2 oncogene product forms a complex with p53 protein and inhibits p53-mediated transactivation. *Cell* 1992, 69:1237–1245.
- Deng C, Zhang P, Harper JW, Elledge SJ, Leder P: Mice lacking p21CIP1/WAF1 undergo normal development but are defective in G1 checkpoint control. *Cell* 1995, 82:675–684.
- Waldman T, Kinzler KW, Vogelstein B: p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res* 1995, 55:5187–5190.
- Halazonetis TD, Kandil AN: Conformational shifts propagate from the oligomerization domain of p53 to its tetrameric DNA binding domain and restore DNA binding to select p53 mutants. *EMBO J* 1993, 12:5057–5064.
- Hupp TR, Meek DM, Midgley CA, Lane DP: Activation of the cryptic DNA binding function of mutant forms of p53. *Nucleic Acids Res* 1993, 21:3167–3174.
- Hupp TR, Sparks A, Lane DP: Small peptides activate the latent sequence-specific DNA binding function of p53. *Cell* 1995, 83:337–345.
- Picksley SM, Vojtesek B, Sparks A, Lane DP: Immunochemical analysis of the interaction of p53 with MDM2: fine mapping of the MDM2 binding site on p53 using synthetic peptides. *Oncogene* 1994, 9:2523–2529.
- Eastham JA, Hall SJ, Sehgal I, Wang J, Timme, TL, Yang G, *et al.*: *In vivo* gene therapy with p53 or p21 adenovirus for prostate cancer. *Cancer Res* 1995, 55:5151–5155.
- Chen J, Jackson PK, Kirschner MW, Dutta A: Separate domains of p21 involved in the inhibition of Cdk and PCNA. *Nature* 1995, 374:386–388.
- Harper JW, Elledge SJ, Keyomasi K, Dynlacht B, Tsai L-H, Zhang P, *et al.*: Inhibition of cyclin-dependent kinases by p21. *Mol Biol Cell* 1995, 6:387–400.
- Luo Y, Hurwitz J and Massague J: Cell-cycle inhibition by independent CDK and PCNA binding domains in p21^{WAF1}. *Nature* 1995, 375:159–161.
- Nakanishi M, Robetorye R, Pereira-Smith O, Smith J: The C-terminal of p21SDI1/WAF1/CIP1 is involved in proliferating cell nuclear antigen binding but does not appear to be required for growth inhibition. *J Biol Chem* 1995, 270:17060–17063.
- Baldin V, Lukas J, Marcote MJ, Pagano M, Draetta G: Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev* 1993, 7:812–821.
- Sherr C: G1 phase progression: cycling on cue. *Cell* 1994, 79:551–555.
- Fähraeus R, Paramio JM, Ball KL, Lain S, Lane DP: Inhibition of pRb phosphorylation and cell-cycle progression by a 20 residue synthetic peptide derived from p16^{CDKN2/INK4A}. *Curr Biol* 1996, 6:84–91.
- Chen J, Saha P, Kornbluth S, Dynlacht BD, Dutta A: Cyclin-binding motifs are essential for the function of p21Cip1. *Mol Cell Biol* 1996, 16:4673–4682.
- Lin J, Reichner C, Wu X, Levine A: Analysis of wild-type and mutant p21^{WAF1} gene activities. *Mol Cell Biol* 1996, 16:1786–1793.
- Russo AA, Jeffrey PD, Patten AK, Massague J, Pavletich NP: Crystal structure of the p27Kip1 cyclin-dependent-kinase inhibitor bound to the cyclin A–Cdk2 complex. *Nature* 1996, 382:325–331.
- Goubin F, Ducommun B: Identification of binding domains on the p21Cip1 cyclin-dependent kinase inhibitor. *Oncogene* 1995, 10:2281–2287.

37. Nakanishi M, Robetorye RS, Adami GR, Pereira-Smith O, Smith J: Identification of the active region of the DNA synthesis inhibitory gene p21^{Sdi1}/CIP1/WAF1. *EMBO J* 1995, 14:555–563.
38. Chen IT, Akamatsu M, Smith ML, Lung FDT, Duba D, Roller PP, *et al.*: Characterization of p21^{Cip1}/Waf1 peptide domains required for cyclin E/Cdk2 and PCNA interaction. *Oncogene* 1996, 12:595–607.
39. Warbrick E, Lane DP, Glover D, Cox L: A small peptide inhibitor of DNA replication defines the site of interaction between the cyclin-dependent kinase inhibitor p21^{WAF1} and proliferating cell nuclear antigen. *Curr Biol* 1995, 5:275–282.
40. Ball KL, Lane DP: Human and plant proliferating-cell nuclear antigen share a highly conserved binding specificity for the p53-inducible gene product p21^{WAF1}. *Eur J Biochem* 1996, 237:854–861.
41. Li B, Tom JY, Oare D, Yen R, Fairbrother WJ, Wells JA, Cunningham BC: Minimization of a peptide hormone. *Science* 1995, 270:1657–21.
42. Hall H, Williams EJ, Moore SE, Walsh FS, Prochiantz A, Doherty P: Inhibition of FGF-stimulated phosphatidylinositol hydrolysis and neurite outgrowth by a cell-membrane permeable phosphopeptide. *Curr Biol* 1996, 6:580–587.
43. Lehman TA, Modali R, Boukamp P, Stanek J, Bennett WP, Welsh JA, *et al.*: p53 mutations in human immortalized epithelial cell lines. *Carcinogenesis* 1993, 14:833–839.
44. Powis G: Signalling targets for anticancer drug development. *Trends Pharmacol Sci* 1992, 12:188–194.
45. Gibbs JB, Oliff A: Pharmaceutical research in molecular oncology. *Cell* 1994, 79:193–198.
46. Gibbs JB, Oliff A, Kohl NE: Farnesyltransferase inhibitors: Ras research yields a potential cancer therapeutic. *Cell* 1994, 77:175–178.
47. Moodie SA, Wolfman A: The 3R's of life: Ras, Raf, and growth regulation. *Trends Genet* 1994, 10:44–48.
48. Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry M, *et al.*: Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* 1994, 370:527–532.
49. Pawson T, Schlessinger J: SH2 and SH3 domains. *Curr Biol* 1993, 3:434–432.
50. Yu H, Chen JK, Feng S, Dalgarno DC, Brauer AW, Schreiber SL: Structural basis for the binding of proline-rich peptides to SH3 domains. *Cell* 1994, 76:933–945.
51. Davies DR, Padlan EA, Sheriff S: Antibody–antigen complexes. *Annu Rev Biochem* 1990, 59:439–473.
52. de Vos AM, Ultsch M, Kossiakoff AA: Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science* 1992, 255:306–312.
53. Kelley RF, O'Connell MP: Thermodynamic analysis of an antibody functional epitope. *Biochemistry* 1993, 32:6828–6835.
54. Clackson T, Wells JA: A hot spot of binding energy in a hormone–receptor interface. *Science* 1995, 267:383–386.
55. Su J-Y, Rempel R, Erikson E, Maller JL: Cloning and characterization of the *Xenopus* cyclin-dependent kinase inhibitor p21^{Xic1}. *Proc Natl Acad Sci USA* 1995, 92:10187–10191.
56. Zhang H, Hannon GJ, Beach D: p21-containing cyclin-dependent kinases exist in both active and inactive states. *Genes Dev* 1994, 8:1750–1758.
57. Renzing J, Hansen S, Lane DP: Oxidative stress is involved in the UV activation of p53. *J Cell Sci* 1996, 109:1105–1112.